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GENETICS OF BACTERIA

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Our work has mainly shifted to DNA transfer in Bacillus subtilis. The main line of work continuing with E. coli is Dr. E.M. Lederberg's study of deletional losses of genes of the galactose complex from exogenotic fragments. Many heterogenotes initially containing fragments that span the entire sequence of galactose genes throw off progeny which have lost parts of that implement. A map of the galactose genes based on the deletion pattern has given a sequence roughly analogous to that established by other methods, but many anomolies remain.

For the B. subtilis work a substantial library of mutants has been accumulated and are routinely tested in all convenient combinations for linkage. Most of the mutants affecting the biosynthesis of aromatic amino acids are linked on the same molecule of DNA. Dr. E.W. Nester has established their linkage order by three- and four-point mapping tests. The genetic sequence of the markers tends to correspond to the biosynthetic steps. However, one intercalated segment within this molecule concerns a step of histidine synthesis. Further more, mutations have been found in which the synthesis of tyrosine is repressed by histidine. The repression phenomenon gives us a unique opportunity of determining whether these controls are exerted by direct combination of the repressor substances with the genic DNA. The well established linkage of markers in the tryptophane pathway has allowed an examination of the molecular conditions for linkage.

Larger DNA molecules containing several linked genes can be sheared either mechanically or enzymatically into smaller segments in which the individual genes remain active, but are no longer linked to one another.

The DNA of B. subtilis is compositionally more heterogeneous than DNA from some other sources. This is manifest by a significant broadening of the DNA band in pycnographic separations in cesium chloride solutions. Spectrophotometric studies on the fractions, together with their thermal denaturation patterns suggest a variation in composition of different genes from about 42-45% GC.

We do not know much about competence whereby properly treated cells obtain the capacity to take up DNA. The competent cells appear when the culture begins to lyse. The competent cells in the population, which rarely but occasionally exceed a level of 1% of the population, have the following properties: they are substantially less sensitive to osmotic shock; they are somewhat retarded in their growth and therefore more resistant to killing by penicillin; they sediment more rapidly and may therefore be larger then the typical cells; and they are rather more sensitive to killing by high temperature or exposure to erythrosin and light. These observations point to the competent cells being atypical in their growth properties, but do not yet lead to a comprehensive picture of their distinction. The competence but not the viability of the cells can be abolished with either periodate or with pronase; this suggests that the receptor substance may be a glycoprotein. Competence is significantly reduced by exposing the cells to glutathione.

SEXUALITY IN E. COLI

F-Prime Strains (Hirota, Sneath, E.M. Lederberg). Some strains of E. coli K 12 had been noted to be highly fertile in an unstable fashion; for the most part they had been isolated by indirect selection (replica plating) for high male fertility. When crossed to Lac$^-$ F$^-$ females some of these gave unstable Lac$^+$ recombinants segregating Lac$^-$ progeny. This process was reminiscent of our previous observations on heterogenosis after transduction of Gal$^+$ by lambda. Further studies indicated that the F particle, normally concerned uniquely with sex-determination, could become associated with a chromosome segment, which it then transferred at conjugation with very high efficiency. Similar findings have been published by Adelberg and Jacob, under the heading of F-episomes.

Attachment Sites for F$^+$ (Richter). Most female strains are infectable with F to become standard F$^+$ males. In such males, F behaves as an extra chromosomal particle which rarely attaches to the chromosome and mediates a conjugal function. Special female strains were found to have sites with a high affinity for infecting F particles, leading to the prompt establishment of a specific Hfr character.

Is F$^+$ Fertility due to Mutation to Hfr? (Lederberg). That some can be attributed to mutation is indubitable, since Hfr mutants are isolated from F$^+$ cultures. However, this effort often fails, and many Hfr clones appear to be highly unstable. Plating of F$^+$ cells into lawns of F$^-$ indicators showed a high incidence of F infection of the recombinants (as reported originally); Hfr F$^-$ progenies on the same plates did not, refuting the suggestion that the transfer of F is by plate superinfection of the cross. That is, the mating recombinant-yielding cell in most F$^+$ X F$^-$ crosses if F-infective, unlike the typical Hfr mutant. However, all intermediate degrees of stability of the F/chromosome association are indicated.

Disinfection of F (Hirota). Hirota had previously shown that F$^+$ cultures of E. coli lost their F$^+$ character when cultivated in the presence of acridine dyes. Examination of small clones in microdrops verified that this was an induced loss and not a selection of spontaneous F$^-$ mutants. While the known reactivity of acridine orange with nucleic acids is suggestive, we could devise no explicit proof that F is DNA or RNA, one of many frustrations at reducing E. coli genetics to chemistry.

Devirilization of Male Cells (Sneath). Acridine orange removes the F particle but does not immediately destroy the male-reactive surface of F$^+$ cells. Conversely, periodate inactivates a male "receptor" but does not attack the F particle, viz. maleness regenerates on further incubation of the treated cells. This clue should have led us to the delineation of a specific receptor substance, but efforts to establish an assay for this, e.g., the blockade of female cells were singularly fruitless.

Genetic Classification of Galactose Mutants (E.M. Lederberg). The λ-gal transduction system was exploited for a study of gene-enzyme relations, initially in collaboration with Kalckar. To make the systems useful for biochemical study by a number of workers, the mutants had to be classified, especially in cis-trans functional tests, and homogenotes and HFT test lysates established. Cistrons relating to each of the enzymatic steps in galactose utilization (as established by Kalckar) were identified.

Gal_K Kinase $Gal + ATP \longrightarrow Gal\text{-}1\text{-}P + ADP$

Gal_T Transferase $Gal\text{-}1\text{-}P + UDP\ Glu \rightleftharpoons Glu\text{-}1\text{-}P + UDP\ Gal$

Gal_E Epimerase $UDP\ Gal \rightleftharpoons UDP\ Glu$

In addition, contrary to then firm ideas, point mutations affecting all three functions were found, and attributed to a regulatory system, now formulated as an "operon"

Biochemical Characterization of Gal mutants (Soffer). In parallel with the cistron tests, a range of mutants was screened for enzymatic defect, mainly by Kalckar's methods. The classification was generally concordant with the cistron test. However, many Gal mutants tend to undergo solution for superimposed mutations of other steps, requiring cautious control of the genotype of strains as actually used for biochemical study.

Deletional Losses from Gal^+ Exogenotes (E.M. Lederberg). The transduced fragments, e.g., in a system $Gal^+ \longrightarrow Gal^- \longrightarrow Gal^+_{ex}/Gal^-$ generally span the whole set of Gal genes. On occasion, clones of such heterogenotes throw progeny that are still heterogenotic, but have a restricted content of Gal genes in the exogenote, the variation resembling segmental deletion of greater or less extent. The deletion pattern has been used to try to map the Gal genes, with partial success. Dr. E. Calef (Pavia) had independently discovered similar deletions and a joint publication is being prepared.

Immunogenetics of E. coli (Orskov and Orskov). Strain K 12 is unsuitable for serological analysis. But Kauffmann and the Orskovs have established an elaborate serotyping scheme for the species E. coli in which recombinational patterns stand out. A systematic survey of serotype strains showed no correlation between serological type and crossability. At first only a few per cent of strains were believed to be fertile, but improved techniques have raised that estimate to a majority, though many strains show a low productivity with some partners. Attempts to map the antigen genes were hindered by many technical difficulties. They were eventually located in the most inaccessible part of the map, and recombination of O, K, and H-determinants established. On the basis of these studies, the Orskovs have subsequently established a unique F-determined antigen which may be equivalent to the male-receptor, and allows another approach to its assay and localization.

Deletions of the Lac region (Cook). Mutants affecting β-galactosidase have played an important role in the development of current ideas on gene-enzyme relationships. Most of these are point mutants affecting one of their functions - in Monod's terminology, y^- (permease) z^- (enzyme structure) i^- (inducibility). Further, some mutants are o^- (operator) regulating the function of the whole segment (operon). A few mutants were shown to behave as deletions, when tested by large scale recombination analysis. It was hoped that the deletions would help in the fine structure mapping of the Lac region, but most of them proved to be very large but coextensive losses pointing to some structural peculiarity in the one strain in which they frequently occur. The deletions have however been particularly useful in physiological studies in Pardee and Jacob's laboratories and in the study of Pl transduction by Luria. A very few small and diagnostically useful deletions were found. One of these has a pure z effect although it encompasses a number of point mutations which have both z and y effects until now attributed to an "operator" segment. The model may be too simple.

Detection of Enzyme Activity of Single Cells and Single Molecules (Rotman). Present studies of enzyme activity treat an ensemble of cells or molecules as if they are homogeneous, and can give only an average picture of particle population. Methods to examine single particles would have wide applications. Such a method has been developed for β-galactosidase involving the use of derivatives of fluorescein in a microscope-fluorometric system. The enzyme releases fluorescent products from a non-fluorescent substrate. The system was originally designed to study single bacteria in the microdroplets but was so successful that its application to single enzyme molecules seemed feasible and was, in fact, accomplished. Enzyme solutions diluted to a concentration of the order of one molecule per sample droplet showed a Poisson distribution of fluorescence intensity in good accord with this expectation, and verifying the turnover number of the enzyme. Another application of the method was to show that thermal inactivation has an all-or-non effect on the activity of single enzyme molecules. On the other hand, a mutant strain of E. coli (D. Perrin) produces enzyme molecules which are uniformly depressed in activity, not a mixture of inactive and fully active molecules.

GENETICS OF SALMONELLA

Control of Flagellar Synthesis in Salmonella (Iino and Lederberg). The alternation of flagellar phases in diphasic Salmonella had been shown to depend on the change of state of a controller gene closely linked or identical with the H_2 antigen determinant. Two complexes of genes have been discovered through the examination of a number of naturally occurring as well as laboratory mutants. One series is linked to the H_1 determinant and includes another factor, Ah_1, necessary for the manifestation of whatever allele is present at the H_1 locus. In addition some factors necessary for the development of any flagella are also linked to H_1. The H_2 complex includes the alleles at H_2 which define which of a number of alternative proteins will be fabricated into the flagellum when the cell is in the H_2 state, the already mentioned phase-2 controller, an Ah_2 factor necessary for any manifestation of H_2, analogous to Ah_1, and finally a Vh_2 factor which regulates the frequency with which the controller oscillates to allow for the alternative manifestation of the H_2 vs.

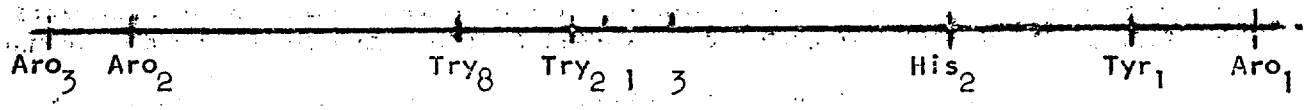
the H_1 antigen. In addition Iino has subsequently characterized other modifiers of the morphology of the flagella and Stocker has demonstrated factors controlling the secondary chemical composition of the flagella, e.g., the methylation of lysine. Altogether at least a dozen distinct genetic functions in the flagellar system have been catagorized. Although complex the system has definite rules which are followed strictly by the overwhelming majority of strains and enable one to understand some genotypic anomalies that have arisen either in the laboratory or in natural populations. For example, one strain of Salmonella paratyphi B, unfortunately very sluggish in its phase variation, has experienced a duplication of the H_1 locus so that derivative strains carrying each of two typical H_1 alleles can be isolated. Furthermore one of the H_1 alleles is serologically indistinguishable from the "1,2" factor which is customarily an allele at H_2. The suggestion that the H_1 and H_2 loci retain some degree of mutual homology is supported by the unique occurrence of a recombinant in which the H_{1b} factor cross over into the H_2 region and behave subsequently as H_{2b}. It would be important to know whether the change of state was strictly a nucleic (nucleotide substitution) phenomenon or whether epinucleic controls are involved. Unfortunately, we can only speculate in the absence of more incisive techniques for the genetic chemistry of Salmonella.

Genetic Analysis of Salmonella Strains by Crossing (Atkinson, Makela, and E.M. Lederberg). Happily superseding our own previous negative reports, Baron and others have succeeded in crossing E. coli with Salmonella and then some Salmonella strains with one another. With the help of this assurance and at first with some of Baron's strains, we hoped to establish crossability among Salmonella strains that might assist in the analysis of the flagellar system. Subsequently we found the F' male determinant to be especially useful for conferring conjugability on the Salmonella strains we were specially interested in. The favorable effect of serial transfer of the F 13 particle from one Salmonella to another in enhancing further fertility suggests some kind of host modification or partial segmental exchange between the F 13 and the indigenous chromosome. However, the F 13 fragment unfortunately has a very low capacity to transfer the chromosome region concerned with antigen determination, and new F-primes must be sought on the same principles to make any headway in immunogenetic analysis.

DNA TRANSFER IN BACILLUS SUBTILIS

Over the past three years we have gradually shifted most of our effort to the system of DNA transfer originally discovered by Spizizen.

Linkage. The preliminary outline of the work was to prepare a number of auxotrophic mutants for comparative studies of their transformation behavior and with the ultimate objective of separating the DNA corresponding to each marker. From past experience with Salmonella transduction, we anticipated that linkage might eventually be found but only after a tedious and extensive search. As it happened the his_2 marker was among the first mutants to be isolated in an indole-requiring strain. Subsequently the two markers were found to be co-transferred at a frequency as high as 70% and the conclusion that they were linked became inescapable. That is, the statistics of transformation leads to the inference that the same DNA molecule carries segments corresponding to a step in the biosynthesis of each of these two amino acids. In further work we have especially concentrated on the properties of this linkage group and have been able to identify and tentatively locate a considerable number of additional markers according to the following map.



It will be noted that most of these markers are concerned with various steps in the biosynthesis of tryptophane, phenylalanine and tyrosine, i.e., the aromatic amino acids. However, additional mutants involved in phenylalanine synthesis have been found which are not closely linked to this segment and the significance of the intercalation of the histidine marker remains enigmatic. These studies have by no means reached the point where we can be sure that the loci indicated are structural genes controlling the amino acid sequence of the corresponding enzymes. We note further the mutation for histidine sensitivity which, being relieved by tyrosine, points to an interrelation or confusion in the repression of biosynthesis of these amino acids. Mutants affecting aromatic amino acid biosynthesis were systematically sought on the expectation that they had a favorable chance of being linked so that the further enlargement of this linkage group is not altogether accidental. (Nester, Schafer and Lederberg)

A second linkage group has recently been found by the application of similar principles - this comprises markers for various early steps in the biosynthesis of arginine.

Breakage of linkage groups by mechanical shear. The "aromatic" linkage group offers an unusual opportunity to examine the molecular conditions for linkage. By treating DNA solutions in a micro-homogenizer the DNA can be degraded to fragments of lower molecular weight whose capacity for linked transfer has been attenuated very much more than the transfer of isolated markers. This is precisely in accord with expectations based on the distribution of the different genes along different segments of an elongated polynucleotide. The same effect can be produced by exposing the DNA for brief intervals to an endonuclease. The studies of enzyme effect on the transforming DNA are especially important as the basis for attempts to establish the net synthesis of biological activity with Kornberg's polymerase system. (Nester and Ganesan).

Efforts at replication of biological activity (net synthesis). (In collaboration with Kornberg and Lehman) (Bodmer). Several previous efforts to establish the replication of transforming activity in the polymerase system using B. subtilis DNA as primer had given only the substantial inactivation of the primer activity. This was attributed to a residue of nuclease in the polymerase preparations and the problem has recently been taken up again with the use of more highly purified preparations of the polymerase. So far, these have been confined to the necessary preparatory work of kinetic studies of the inactivation of the transforming activity. The new preparations have shown the surprising effect of considerable reduction in the viscosity (characteristic chain length?) with now inappreciable losses of the activity of the primer material.

Molecular fractionation of B. subtilis genes (Ganesan). The DNA of B. subtilis is compositionally more heterogeneous than DNA from many other bacterial sources. This is manifest by significant broadening of the DNA band in pycnographic separations in cesium chloride solutions. Fractions corresponding to the different buoyant densities have been found to show substantial enrichment of transforming activity for various markers. Spectrophotometric studies on the fractions together with their thermal denaturation patterns suggest a variation in composition of different genes from about 42-45% GC. These findings have made it possible to achieve a substantial fractionation of DNA for specific genetic activities and to allocate different markers to different segments of the variation in GC content. However, the various markers of the "aromatic" linkage group appeared to be relatively uniform in their GC content. Thermal studies suggest that some molecules may be more regionally variable since under certain conditions of denaturation DNA fractions are found which have a buoyant density intermediate between that of the native and the denatured DNA and which have retained most of their biological activity. We would tentatively interpret these intermediate forms as DNA molecules which are partially denatured at the critical temperature owing to a lower GC content in certain segments while the marker genes, having a higher GC content, remain in the intact double stranded condition. Particularly if physico-chemical studies bear out this interpretation of heavy denaturation, such preparations may be particularly useful for differential enzymatic degradation and resynthesis.

Isolation of Nuclei. There is little direct evidence whether the entire DNA of the bacterial genome (which would comprise about 300 of the molecules as we now isolate the DNA) is a single extensive polynucleotide chain or is segmented by non-polynucleotide nodes. The exquisite sensitivity to shear of long polynucleotide molecules in solution might account for the molecular weight of the actual preparations as an artefact of extraction. Some preliminary studies have been made on the possibility of the preliminary extraction of B. subtilis DNA in the form of intact nuclear bodies which would permit them to be dissolved under the gentlest conditions possible in a search for larger molecular segments. Such segments might also be detected by virtue of an augmentation of the apparent linkage between markers not now recognized as situated on the same DNA molecule. The extraction of protoplasts in the presence of spermine has given rise to nuclear bodies whose DNA content is apparently protected by the precipitation of salt formations with the spermine base. However, these bodies have been difficult to redissolve and when this is accomplished give little evidence of the persistence of any larger DNA molecules than in the usual preparations. The procedure for the isolation of such nuclear bodies should however be invaluable for studies on the dynamics of transfer of transforming DNA from the external milieu of the transformed cell to the integrated chromosome. (Ganesan)

Segregation. Some elementary questions in the immediate history of transferred markers have been studied. A small percentage of cells exposed to DNA from two distinct donors will give rise to mixed clones manifesting the diverse markers in separate subclones (digressive co-transfer). These clones are, however, too infrequent to materially affect calculations on transforming kinetics. The same can be said for the frequency of digressive clones arising from transforming experiments involving several markers from the same donor parent. From time to time transformant clones have been seen which appear to be persistently segregating different markers of the donor parent ro markers of both the donor and recipient parent. These would be of special interest if they could be established as heterogenotic clones by analogy with the transductional system. However, B. subtilis has been found to be considerably less reliable in its response to conventional streaking and plating methods for the separation of pure clones than are the enteric bacteria; and subject to this caution it is not possible to make firm conclusions about the persistence of the heterogenotic state. (E.M. Lederberg).

Dr. Bruce Stocker spent a few months in the laboratory familiarizing himself with the B. subtilis system and making some preliminary experiments on the transfer of motility to single cells, partly in order to answer the questions of the previous paragraph. His work along these lines is continuing in London.

Competence (Fradkin). One of the most mysterious aspects of DNA transfer in B. subtilis remains the mechanism of competence, whereby properly treated cells obtain the capacity to take up DNA. The competent cells appear at the stage in the growth cycle of the organisms at which the culture is beginning to undergo spontaneous lysis. The competent cells in the population, which

rarely, but occasionally exceed a level of 1% of the population, have the following properties. They are substantially less sensitive to osmotic shock; they are somewhat retarded in their growth and therefore more resistant to killing by penicillin; they sediment more rapidly and may therefore be larger than the typical cells; and they are rather more sensitive to killing by high temperature or exposure to erythrosin and light. These observations point to the competent cells being atypical in their growth properties, but do not yet lead to a comprehensive picture of their distinction. The competence but not the viability of the cells can be abolished with either periodate or with pronase; this suggests that the receptor substance may be a glyco-protein. Competence is significantly reduced by exposing the cells to glutathione.

EXOBIOLOGY

Molecular biology of the planets. Studies on the genetic functions of DNA in microorganisms also form the conceptual basis of a program of investigation of other planets of the solar system for manifestations of life. Very high priority has accordingly been given a) to the detection of microorganisms as indicators of life on Mars, b) the determination whether such organisms, if they exist, have the same nucleic acid and protein bases as do all terrestrial organisms, and c) the urgency of extreme caution to avoid the inadvertant transfer of terrestrial organisms to other habitable sites. The implementation of such a program is of course the responsibility of the NASA and special laboratory with an engineering and analytical orientation has been established under that agency's sponsorship for the purpose. However, it has been important not to isolate these activities from other basic research. Not only does exobiology depend on the most incisive and perspective understanding of terrestrial biochemistry and genetics, but the instrumentation developed for exobiological study is being designed to take full advantage of possible applications in the terrestrial laboratory. Fortunately the management of the exobiology work is now in the expert hands of Dr. E.C. Levinthal affording the best opportunity for mutual help at a scientific and technical level.

Under current development, hopefully for flight during the 1964 opposition, is an instrument designed to be landed on Mars, sample its soil, and look for life as it might be manifest by the enzymatic activity of the soil. The principles of sensitive detection on which this system is based derives directly from Dr. Rotman's work on the detection of single molecules of β-galactosidase with fluorogenic substrates.